Cell, Vol. 84, 723–734, March 8, 1996, Copyright ©1996 by Cell Press

Wiskott–Aldrich Syndrome Protein, a Novel Effector for the GTPase CDC42Hs, Is Implicated in Actin Polymerization

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Summary

The Rho family of GTPases control diverse biological processes, including cell morphology and mitogenesis. We have identified WASP, the protein that is defective in Wiskott–Aldrich syndrome (WAS), as a novel effector for CDC42Hs, but not for the other Rho family members, Rac and Rho. This interaction is dependent on the presence of the G protein–binding domain. Cellular expression of epitope-tagged WASP produces clusters of WASP that are highly enriched in polymerized actin. This clustering is not observed with a C-terminally deleted WASP and is inhibited by coexpression with dominant negative CDC42Hs-N17, but not with dominant negative forms of Rac or Rho. Thus, WASP provides a novel link between CDC42Hs and the actin cytoskeleton, which suggests a molecular mechanism for many of the cellular abnormalities in WAS. The WASP sequence contains two novel domains that are homologous to other proteins involved in actin organization.

Introduction

Members of the Ras superfamily of small GTPases play essential roles in the regulation of diverse cellular functions such as growth control, vesicular transport and cytoskeletal organization (Nobes and Hall, 1994). These proteins function as binary molecular switches by cycling between the active GTP-bound state and the inactive GDP-bound state (Boguski and McCormick, 1993). The Rho-like GTPases include RhoA, RhoB, and RhoC, Rac1 and Rac2, CDC42Hs, RhoG, and TC10. Members of this subfamily share ~30% sequence identity with other Ras-related proteins and >50% sequence identity with each other (Nobes and Hall, 1994).

Over the last few years, it has become apparent that members of the Rho subgroup are key elements in the dynamic organization of the actin cytoskeleton. Dissection of the function of Rho GTPases in mammalian cells identified Rac as a mediator of growth factor–induced lamellipodia formation (Ridley et al., 1992) and Rho as a regulator of stress fibers (Ridley and Hall, 1992). In yeast, CDC42 was shown to be involved in bud formation (Johnson and Pringle, 1990) and in mammalian cells, CDC42Hs triggers induction of microspikes and filopodia, followed by the formation of lamellipodia (Kozma et al., 1995; Nobes and Hall, 1995). In addition, CDC42Hs, Rac, and Rho also control the formation of specialized adhesion complexes (Nobes and Hall, 1995). Lamellipodia and stress fibers induced by CDC42Hs are inhibited by the dominant negative Rac-N17 and by C3 exoenzyme, respectively, suggesting that the GTPases CDC42Hs, Rac, and Rho can be organized in a linear cascade to control the actin cytoskeleton (Nobes and Hall, 1995). Similarly, a GTPase cascade involving Bud1 and CDC42 is implicated in actin reorganization during budding in Saccharomyces cerevisiae (Zheng et al., 1995). The GTPase targets involved in mitogenesis and the control of the actin cytoskeleton are currently unknown. An effector candidate for Rac and CDC42Hs was recently identified as the serine/threonine kinase PAK65, which is related to yeast STE20 (Manser et al., 1994; Martin et al., 1995). Whereas PAK65 kinase activity is stimulated by GTPase-dependent autophosphorylation, no link to the cytoskeleton has yet been established. Other potential players include phosphoinositol (PI) 5-kinase activated by Rho (Chong et al., 1994), PI 3-kinase activated by CDC42Hs (Zheng et al., 1994), and phospholipase A2 activated by Rac (Peppelenbosch et al., 1995) to produce PIP_2, PIP_3, and arachidonic acid, respectively. These lipid products may modulate the activity of actin-binding proteins involved in the control of actin polymerization. Recent data support the involvement of Rho-like GTPases in multiple pathways, including the stress-activated (JNK/SAPK) signaling cascade (Minden et al., 1995; Coso et al., 1995), stimulation of DNA synthesis, transcriptional activation by serum response factor (Hill et al., 1995), and cell transformation (Qiu et al., 1995a).

In this study, we report the isolation of a novel effector for CDC42Hs and identify it as the Wiskott–Aldrich syndrome (WAS) protein (WASP). The WASP gene was recently isolated by positional cloning and was shown to be mutated in WAS patients (Derry et al., 1994). WAS is an X-linked recessive disorder characterized by the triad of thrombocytopenia, recurrent infections due to defects in T and B cell function, and eczema (Ammann and Hong, 1989). The cellular defects in WAS patients are limited to hematopoietic lineages and include cytoskeletal abnormalities of T cells and platelets (Molina et al., 1992), failure of B cells to respond to polysaccharide antigens, and defective chemotaxis in neutrophils (Ochs et al., 1980), suggesting that a defect in the organization of actin cytoskeleton may lie at the basis of the syndrome. Approximately 50 different naturally occurring WASP mutations have been identified in patients with classic or attenuated forms of the disease, with some correlation between the various missense, nonsense, and splice site mutations and clinical severity (Derry et al., 1995a; Kolluri et al., 1995; Kwan et al., 1995; Villa et al., 1995; Zhu et al., 1995; Wengler et al., 1995).

The WASP sequence encodes a proline-rich protein and was shown recently to bind NCK (Rivero-Lezcano

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et al., 1995), an SH3/SH2 adaptor protein (Lehmann et al., 1990). The WASP sequence also contains a putative nuclear localization signal and an acidic C-terminal domain (Derry et al., 1994). By cell fractionation, WASP has been shown to be cytosolic with low levels in the nucleus (Rivero-Lezcano et al., 1995).

Here we report that WASP has a GTPase-binding site that is similar to those found in Rac/CDC42Hs effector proteins and show that it interacts specifically with activated CDC42Hs. In different cell types, WASP localizes in clusters that are enriched in polymerized actin and are regulated by CDC42Hs. In addition, we also identified novel domains on WASP that are conserved among proteins that are involved in the organization of the actin cytoskeleton. Our study provides a link between a member of the Rho family, its effector WASP, and the control of actin polymerization and suggests a possible mechanism for defects of WAS that are associated with actin cytoskeleton abnormalities.

Results

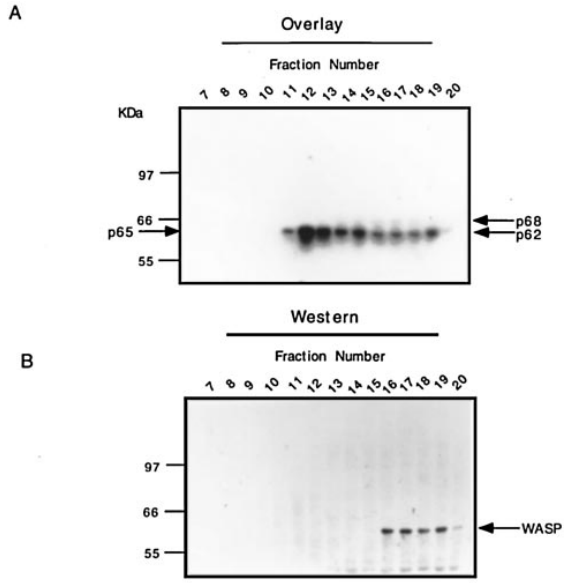
A 62 kDa CDC42Hs-Binding Protein in Neutrophils Is Identical to WASP

By utilizing an overlay method, we recently identified three proteins in human neutrophil cytosol that bind the activated GTPase CDC42Hs (Martin et al., 1995). When fractions of cytosol, separated on a Mono Q column, are probed on a filter with CDC42Hs preloaded with [γ-^{32}P]GTP, three bands of apparent molecular sizes 62, 65, and 68 KDa are detectable (Figure 1A). As previously reported, p65 is a member of the PAK family, whose kinase activity is stimulated by Rac or CDC42Hs (Martin et al., 1995). We have now isolated the p62 protein, by sequential column chromatography, and have determined the sequence of a tryptic peptide. Interestingly, 16 of 17 amino acids of this peptide are identical to amino acid sequence 288–304 of WASP (Figure 1C). The discrepant residue, a glutamate in p62 and a glutamine in WASP, is most likely due to an error in peptide sequencing since a glutamine is conserved in the mouse WASP gene (Derry et al., 1995b) and since no E/Q sequence polymorphism has been observed in numerous human WASP (hWASP) alleles (Derry et al., 1995a).

To confirm that p62 and WASP are identical proteins, we probed the filter of fractionated cytosol protein shown in Figure 1A with anti-WASP antibody and subsequently with anti-PAK65 antibody. The p62 protein eluted in fractions 16–20 and was recognized by anti-WASP antibody (Figure 1B), whereas p65 eluted in fractions 11–15 and was detected with anti-PAK65 antibody (data not shown). These results further establish that p62 and WASP are the same protein.

WASP Contains a CDC42Hs-Binding Domain

To identify proteins that contain the putative GTPase (Rac/CDC42Hs)-binding domain (GBD) of PAK (Manser et al., 1994), we conducted a search through the database using the GBD sequence of human PAK65 (hPAK65) (74-EISPPSDFEHTIHVGFDTVTG-95) as the query. Interestingly, in addition to PAK-related proteins such as STE20, CLA4, and Shk1 (Cvrckova et al., 1995; Marcus et al., 1995; Ramer and Davis, 1993), the GBD was also found in several proteins that are not serine/threonine kinases, including WASP (Figure 2A). By comparing the putative GBD of PAK-related genes from human to yeast, a consensus of 14 amino acids can be derived (Figure 2A). The WASP GBD sequence (amino acids 238–257) shares 10 of the 14 residues of the consensus and six of the eight amino acids that are invariant in the other proteins (marked by asterisks in Figure 2A).



C

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p62:        K L I Y D F I E D E G G L E A V R
WASP: 287   K L I Y D F I E D Q G G L E A V R 304
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Figure 1. Fractionation and Protein Analysis of Neutrophil Cytosol

(A) Neutrophil cytosol (10 ml; ~10 mg of protein) was applied on a Mono Q column and eluted with a 30 ml salt gradient (0–0.5 M NaCl). Collected fractions were analyzed by the overlay assay and probed with [γ-^{32}P]GTP–CDC42Hs.

(B) The same filter used in (A) was washed and subsequently was subjected to immunoblot analysis with rabbit polyclonal antiserum developed against recombinant WASP (amino acids 48–321).

(C) Sequence homology between a peptide obtained from enzymatically digested p62 and WASP. Identical amino acids are shown in bold.

WASP Binds Preferentially to CDC42Hs

Having identified p62 as WASP, we next studied the binding specificity of a WASP–glutathione S-transferase (GST) fusion protein for the GDP- or GTP-bound forms of members of the Rho GTPase family. WASP–GST, containing amino acids 48–321 that include the putative GBD (amino acids 238–257) but exclude most of the proline-rich region (Derry et al., 1994), interacted only with the GTP-bound form of CDC42Hs but did not interact with Rac or Rho (Figure 2B). After longer exposure of the film, a very weak interaction was observed for GTP-bound Rac1, but not for Rho. In contrast, hPAK65 interacts strongly with the GTP-bound forms of both Rac1 and CDC42Hs. No binding was detected for GST

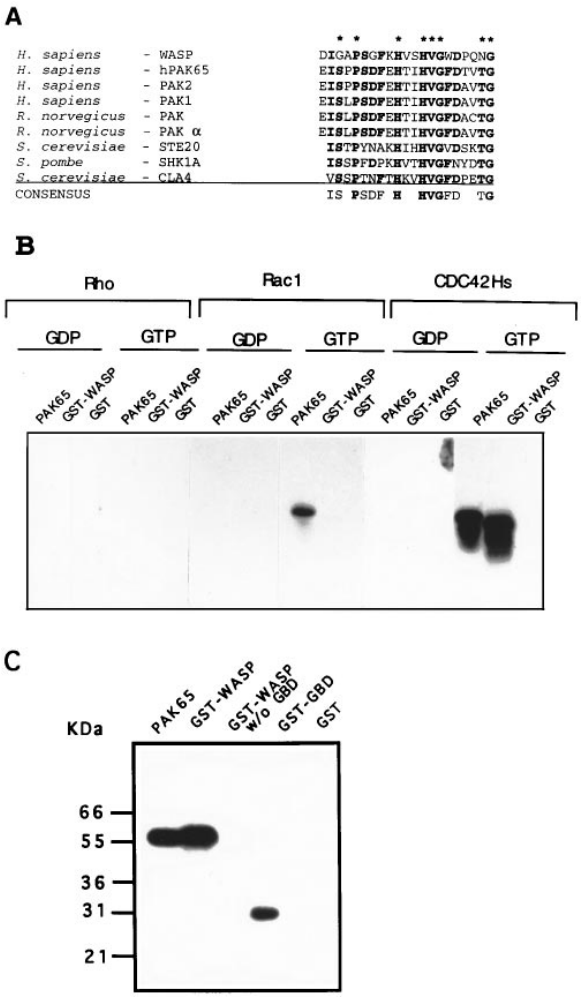

Figure 2. WASP Binds to Activated CDC42Hs via Its GBD

(A) The putative GBDs in WASP- and PAK-related proteins are compared. Conserved amino acids are shown in bold, and invariant amino acids are indicated by asterisks.

(B) Recombinant proteins hPAK65, GST–WASP, and GST (2–3 μg) were analyzed by the overlay assay with the indicated GTPase preloaded either with [γ-^{32}P]GTP or [β-^{32}P]GDP.

(C) hPAK65, GST–WASP, GST–WASP without the GDB, and GST–GBD recombinant proteins were analyzed by the overlay assay with CDC42Hs preloaded with [γ-^{32}P]GTP.

alone. These results indicate that WASP is primarily an effector for CDC42Hs.

The GBD Is Sufficient and Essential for CDC42Hs Binding

To evaluate the role of the putative GBD, we have deleted the GBD from the WASP–GST plasmid (amino acids 237–257) and constructed a GST fusion plasmid that contains the WASP–GBD (amino acids 235–268). As demonstrated by overlay assay (Figure 2C), the fusion protein WASP–GST without GBD did not bind activated CDC42Hs, while the small peptide including the GST–GBD fusion protein did bind. These experiments confirm that the GBD of WASP is both necessary and sufficient for CDC42Hs binding.

Expression of WASP Induces Ectopic Actin Polymerization

To determine the subcellular distribution of WASP, we performed transient transfection of normal rat kidney (NRK) epithelial cells with FLAG epitope–tagged WASP. Examination of the cells 40–48 hr posttransfection showed cytoplasmic staining in most of the transfected cells. In a small minority (<5%), only the nucleus was labeled. Cells expressing low levels of WASP displayed a punctate pattern of WASP staining, whereas higher expressing cells showed extended cluster formation of WASP-containing particles (Figure 3A). Dual labeling with phalloidin, a fungal toxin specific for filamentous actin (F-actin), revealed a striking colocalization of WASP with polymerized actin, both for the small WASP-containing particles and the larger clusters (Figure 3B). This costaining was not seen for all actin structures in the cell; none of the endogenous stress fibers, for instance, were clearly labeled with the anti-FLAG antibody, although all regions labeled with anti-FLAG antibody also contained F-actin. Transfected cells expressing very high levels of WASP showed a reduction in the number of stress fibers, indicating a disruption of the endogenous actin cytoskeleton.

To determine the region of WASP responsible for actin cluster formation, we transfected cells with a construct containing a 443 amino acid, C-terminally truncated WASP (WASPΔC). In WASPΔC-transfected cells, the fluorescent labeling was much more uniformly distributed; punctate staining throughout the cytoplasm could only be detected in cells with high levels of expression (Figure 3C), and colocalization with polymerized actin could not be unambiguously detected (Figure 3D). Similar results were obtained using transient transfection of monkey COS7 cells (data not shown). These results suggest that WASP C-terminal 59 amino acids are implicated in the control of actin polymerization or localization of WASP to actin-containing structures, or both. Alternatively, this deletion could have an effect on WASP folding, thereby altering its function. Experiments with the reciprocal construct, containing only the C-terminal 60 amino acids, are in progress.

Jurkat T cells, which normally express the WASP gene (Derry et al., 1994), may be more physiologically relevant for in vivo studies than epithelial cell lines such as NRK or COS cells, which do not express endogenous WASP. Therefore, we transfected the two epitope-tagged WASP constructs into Jurkat cells and studied the localization of the proteins. Although these cells have a smaller cytoplasmic compartment and are much rounder than COS7 or NRK cells, colocalization of WASP with F-actin was still apparent, with large punctate areas of labeling in the cytoplasm (Figures 3E and 3F). The truncated WASPΔC yielded cytoplasmic labeling with no evidence for colocalization with actin (data not shown).

Functional Interaction of WASP with CDC42Hs

We next performed microinjection studies with WASP expression plasmids in porcine aortic endothelial (PAE) cells. As was found in the transfection studies, cells expressing low levels of WASP displayed punctate la-

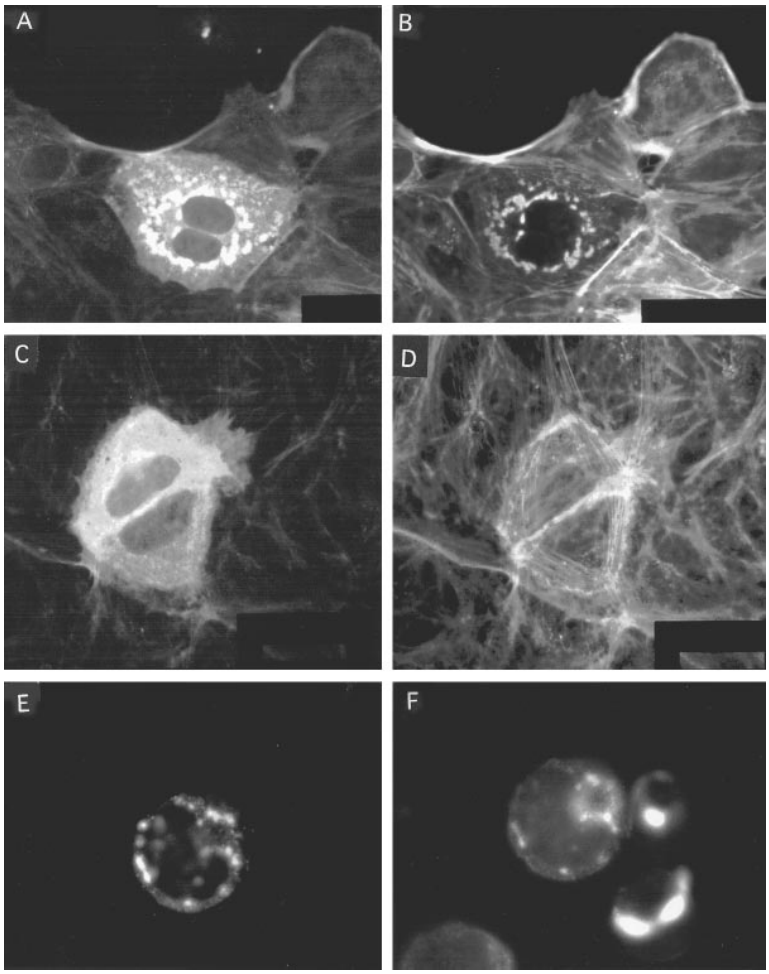

Figure 3. Transient Expression of Transfected WASP Constructs in Rat NRK Cells and in Jurkat Cells

(A–D) NRK cells. (E and F) Jurkat cells. Cells labeled with anti-FLAG antibody (A, C, and E) and simultaneously stained with phalloidin (D, B, and F) were photographed in a standard fluorescence microscope. Cells in (A) and (B) and in (E) and (F) were transfected with a full-length epitope-tagged WASP construct, while for the cells in (C) and (D), we used a truncated WASP (WASPΔC) missing the 59 C-terminal amino acids. Fluorescence micrographs compare anti-FLAG (A, C, and E) and phalloidin staining (D, B, and F).

beling of the cytoplasm, while cells expressing higher levels of WASP contained extended clusters of WASP-rich particles (Figure 4A), which were strongly enriched in F-actin (Figure 4B). Expression of WASPΔC generated a much more diffuse distribution of WASP and greatly diminished formation of extended clusters (Figure 4C).

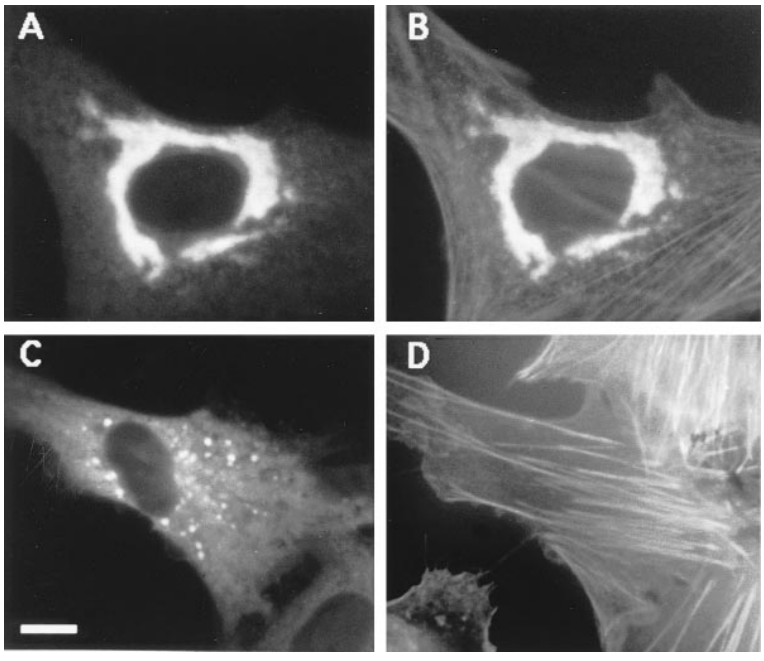

Figure 4. Microinjection of WASP Plasmids into PAE Cells

Expression of WASP (A and B) and of WASPΔC (C and D) leads to different patterns of fluorescence when anti-FLAG (A and C) and phalloidin staining (B and D) are compared. Bar represents 10 μm.

Furthermore, the relatively few clusters were not enriched in F-actin (Figure 4D).

To examine the relation between WASP cluster formation and actin polymerization further, we microinjected cells with WASP expressing plasmids and incubated them with a low concentration (0.1 μg/ml) of cytochalasin D (CD) shortly after injection. This treatment caused strong inhibition in the formation of extended clusters (compare Figures 5A and 5C), suggesting that the clustering process is dependent on actin polymerization. Interestingly, once the extended clusters are formed, the polymerized actin that localizes to these clusters is strongly resistant to CD treatment at concentrations up to 1 μg/ml for 45 min (data not shown).

To study the functional interaction of WASP with Rho family GTPases, we coexpressed WASP with the dominant negative constructs CDC42Hs-N17, Rac1-N17, and RhoA-N19. CDC42Hs-N17 strongly inhibited extended cluster formation of WASP (Figure 6B), while Rac1-N17 and RhoA-N19 had no effect (Figures 6C and 6D), indicating that CDC42Hs is essential for WASP clustering.

We further explored these in vivo interactions by coexpressing WASP with constitutively active forms of these GTPases: CDC42Hs-V12, Rac1-V12, or RhoA-V12. Expression of CDC42Hs-V12 induced extensive lamellipodia and promoted highly characteristic bundling of stress fibers in PAE cells (Figure 7A), consistent with earlier observations indicating that CDC42Hs activates a GTPase cascade comprised of Rac and Rho (Nobes and Hall, 1995). Expression of WASP, even at low levels, strongly inhibited this phenotype (Figure 7B). Expression of constitutively active Rac1-V12 in PAE cells caused a very similar phenotype to that of CDC42Hs-V12, except that stress fiber bundling was less marked (Figure 7C). WASP at low expression levels did not interfere with this phenotype (left cell in Figure 7D), but at expression levels high enough to lead to extensive clustering WASP did inhibit Rac-induced lamellipodia formation (right cell in Figure 7D). Expression of constitutively active RhoA-V14 in PAE cells caused stress fiber formation and cell contraction (Figure 7E), similar to the phenotype induced by RhoA-V14 in other cell types (Ridley and Hall, 1992). Coexpression of WASP, even at high levels, did not have any effect on the RhoA-V14 phenotype (Figure 7F). Taken together, these in vivo results are consistent with the specific in vitro interaction of WASP with CDC42Hs, its much weaker binding to Rac, and its lack of interaction with Rho.

Putative Domains Present in WASP and Other Proline-Rich Proteins

Having identified a link between WASP and F-actin, we speculated that WASP may also contain sequences that represent interaction sites for common cellular components implicated in actin organization and that these domains could be conserved in other proteins. Thus, we compared the amino acid sequence of WASP, excluding the polyproline sequences, with the amino acid and nucleotide sequences of the nonredundant sequence database and with the expressed sequence tag database dbEST using the BLASTP and TBLASTN programs (Stephen et al., 1990). In addition to the GBD, we identified two distinct sequences in WASP in the N-terminal and in C-terminal regions of WASP that are conserved in several proline-rich proteins known to be involved in the organization of actin cytoskeleton (Figure 8A). Because these regions were originally identified in WASP, we propose that these putative domains be termed WASP homology 1 and 2 (WH1 and WH2) domains. The domain structures of proteins that contained WH1 and WH2 are shown in Figure 8B. WH1 is situated near the N-terminus of the identified proteins, followed by a long repetitive polyproline sequence. In contrast, the WH2 motif follows a polyproline sequence located near the N-terminus. A

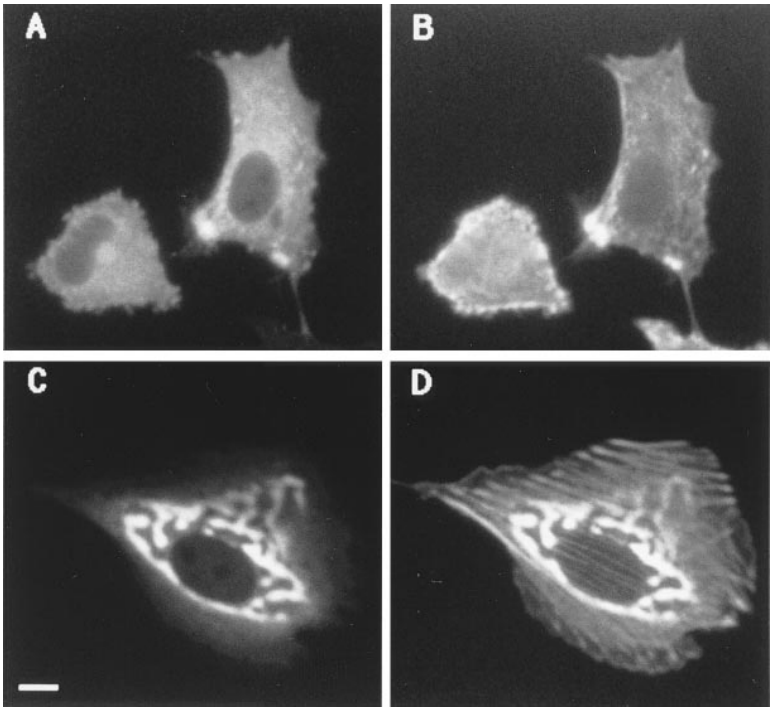

Figure 5. WASP–Actin Cluster Formation Is Inhibited by CD

Expression of WASP in the presence (A and B) and absence (C and D) of CD. Cells in (A) and (B) were injected with the full-length epitope-tagged WASP cDNA. The cells were treated 2 hr after injection with CD at 0.1 μg/ml for an additional 3 hr. Fluorescence micrographs compare anti-FLAG staining (A and C) and phalloidin staining (B and D). Bar represents 10 μm.

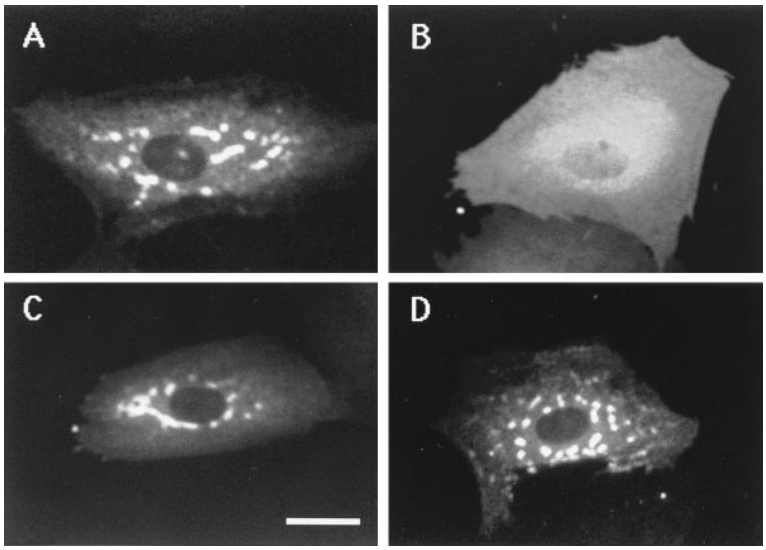

Figure 6. WASP Cluster Formation Is Inhibited by Coexpression of Dominant Negative CDC42Hs-N17

Fluorescence micrographs of anti-FLAG staining of cells expressing WASP alone (A), coexpressing WASP with CDC42Hs-N17 (B), with Rac1-N17 (C), and with RhoA-N19 (D). Bar represents 25 μm.

polyproline protein from S. cerevisiae (YSCLAS17) reveals a striking structural similarity to WASP and contains the WH1 in the N-terminus and polyproline sequences followed by WH2 in the C-terminus (Figure 8B).

Discussion

To elucidate the signaling pathways in which the Rho GTPases are involved, we searched for novel GTPase effectors. We have isolated a 62 kDa protein (p62) that interacts with the activated form of CDC42Hs and have identified it as the product of the gene mutated in WAS (Derry et al., 1994). Moreover, we have established a functional connection between CDC42Hs, WASP, and actin polymerization. The CDC42Hs–WASP–F-actin interaction establishes a link for a Rho family GTPase with the actin cytoskeleton and also provides an indication of the function for WASP, laying the basis for a molecular understanding of the clinical syndrome.

Transfection and microinjection of WASP-expressing

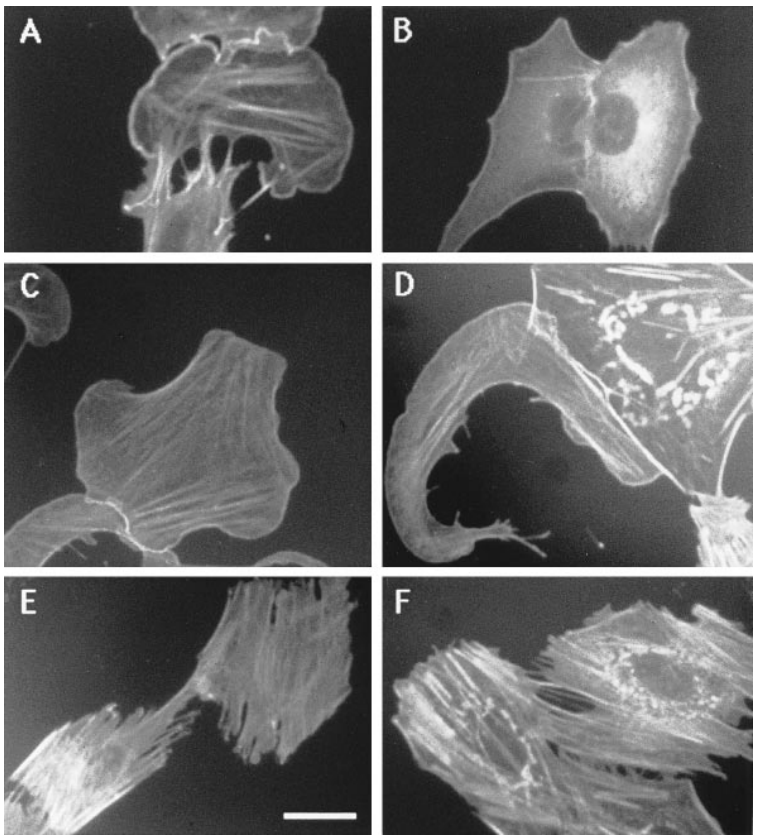

Figure 7. Expression of WASP Interferes with CDC42Hs Function in PAE Cells

Fluorescence micrographs of phalloidin staining of cells expressing CDC42Hs-V12 (A), CDC42Hs-V12 and WASP (B), Rac1-V12 (C), Rac1-V12 and WASP (D), RhoA-V14 (E), and RhoA-V14 and WASP (F). Bar represents 25 μm.

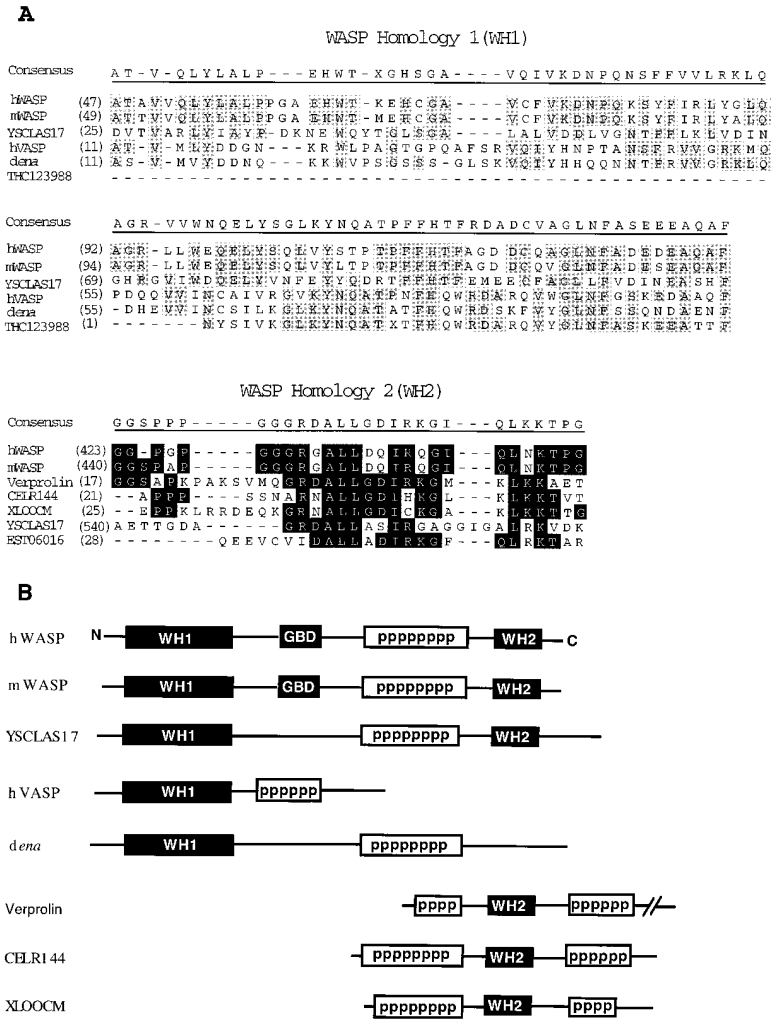

Figure 8. Sequence Comparison

(A) Alignment of the putative WH1 and WH2 domains of amino acid sequences of hWASP, mouse WASP (mWASP), S. cerevisiae proline-rich protein YSCLAS17 (NCBI accession number 1101757), human VASP (hVASP) (NCBI accession number 1085412), Drosophila ENA (dena) (NCBI accession number 755821), verprolin (NCBI accession number 871535), THC123988 (dbEST), proline-rich Caenorhabditis elegans protein CELR144 (NCBI accession number 746496), proline-rich Xenopus laevis protein XLOOCM (NCBI accession number 64956), and EST06016 (dbEST). Alignment was produced using the Laser Gene program. Stippled boxes indicate residues that are identical in at least two members of the group.

(B) Domain structure of proteins containing the WH1 and WH2 domains. The relative positions of WH1, WH2, polyproline sequences, and GBD are compared among the above proteins. Proline-rich regions (PPPPPPPP) represent long stretches of 5–16 consecutive prolines.

plasmids into different cell types consistently demonstrated that WASP has a profound effect on actin polymerization. WASP expression induces the formation of F-actin-rich particles or, in overexpressing cells, dense aggregates of WASP and polymerized actin. These particles are likely to contain new sites of F-actin nucleation. The precise nature of these WASP-containing particles and clusters is not yet clear, however; so far, we have not observed any colocalization with markers for various vesicular compartments. In addition, WASP clustering was not inhibited by the microtubule depolymerizing drug nocodazole (our unpublished data). In contrast, treatment with CD disrupted the formation of WASP clusters, suggesting the involvement of an actomyosin-based process in the formation of these structures.

We have shown that the regulation of WASP–actin cluster formation is mediated by the Rho family GTPase CDC42Hs. The Rho family GTPases have been previously implicated in distinct dynamic processes involving the actin cytoskeleton: the formation of filopodia and lamellipodia by CDC42Hs and Rac, respectively, and the assembly of focal adhesions and stress fibers by Rho (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995; Chant and Stowers, 1995). Our in vitro studies reveal that WASP binds specifically to the GTP-bound form of CDC42Hs, but not to activated Rac or Rho. Based on sequence alignment, we have identified a putative GBD and derived the consensus sequence PxxxxHxxHVGxxxxxG. Furthermore, studies with a GBD-deleted WASP construct and with a GST–GBD-only construct demonstrate that the 21 amino acids of the GBD are necessary and sufficient for WASP–CDC42Hs interaction. The specificity and biological relevance of this interaction are further demonstrated by studies of in vivo coexpression of WASP with dominant negative mutant forms of the GTPases. Dominant negative CDC42Hs-N17 interferes with WASP cluster formation while WASP-induced cluster formation is not inhibited in cells expressing Rac-N17 or Rho-N19.

Further, coexpression of WASP with the constitutively active mutant protein CDC42Hs-V12 disrupts lamellipodia formation in these cells. This inhibition is observed in cells expressing very low levels of WASP. In contrast, inhibition could only be observed at high expression levels of WASP in cells coexpressing Rac-V12, suggesting that WASP is predominantly a target of CDC42Hs. The observation that WASP inhibits lamellipodia formation in PAE cells expressing the constitu-

tively active CDC42Hs-V12 is most readily explained by assuming that CDC42Hs normally activates Rac and that overexpressed WASP acts in a dominant negative fashion by sequestering activated CDC42Hs.

The clinical features of WAS also support a link between WASP function and the actin cytoskeleton. The most common clinical manifestation is severe thrombocytopenia. One possible mechanism for this could be that mutations in the WASP gene lead to abnormal cytoskeletal rearrangement in the megakaryocyte, which results in defective platelet formation. Abnormalities of platelet shape or surface features are recognized by the spleen, which sequesters and destroys these platelets, thereby releasing abnormal microplatelets into the circulation. After splenectomy, circulating platelets are restored to normal size and numbers (Mullen et al., 1993).

Profound immunodeficiency involving B and T cells and recurrent infections are associated with the syndrome (Ochs et al., 1980). Morphological and biochemical studies have suggested that the underlying defect in these phenomena also may involve the actin cytoskeleton (Molina et al., 1992, 1993). Scanning electron microscopy of T cells from WAS patients has shown that microvilli are decreased in size and density compared with normal (Kenney et al., 1986; Molina et al., 1992). This abnormal feature has been proposed to reflect a fundamental defect in the T cell cytoarchitecture. Furthermore, the failure of WAS B cells to respond to polysaccharide antigens (Ochs et al., 1980) and of WAS T cells to respond to immobilized anti-CD3 monoclonal antibody (Molina et al., 1993) is also consistent with an underlying transmembrane signaling defect linked to an abnormality in the actin cytoskeleton. Recognition of mitogenic stimuli is likely to require cell polarization mediated by actin cytoskeletal rearrangements. This hypothesis is supported by the observation that actin bundling is necessary for T cell activation by anti-CD3 (Parsey and Lewis, 1993). It is interesting to note in this respect that CDC42Hs is also required for the polarization of T cells toward antigen-presenting cells (Stowers et al., 1995). The role of CDC42Hs in filopodia formation has been previously demonstrated by microinjection experiments and shown to be independent of Rho and Rac (Kozma et al., 1995; Nobes and Hall, 1995). Thus, WASP may fulfill two roles in T cells, to stabilize microvillus projections and to transmit signals to the cytoskeleton. Taken together, the effects of WASP on the cytoskeleton and the specific interaction with CDC42Hs provide an explanation for the T cell abnormalities observed in WAS.

WASP expression was originally found to be restricted to cells of lymphoid, erythroblastic, and megakaryocytic origin (Derry et al., 1994). We have now also detected WASP/p62 in neutrophil and macrophage cytosol, which raises the possibility that WASP plays a role in the organization of the phagocyte cytoskeleton. It has been reported that chemotactic responses of neutrophils from WAS patients are abnormal (Ochs et al., 1980), potentially as a direct result of a defect in WASP-regulated actin polymerization. In this regard, it is interesting to note that some chemokines signal through G protein–coupled receptors. In the yeast S. cerevisiae, the G protein–coupled pheromone receptor signals to both the nucleus and the cytoskeleton through CDC42 (Simon et al., 1995), while in mammalian cells, the known upstream effectors for CDC42Hs, bradykinin, thrombin, and fMLP, all signal through G protein receptors (Kozma et al., 1995; Teo et al., 1995; Knaus et al., 1995). Thus, an apparently conserved signaling pathway in yeast and mammals involves G protein–coupled receptor, CDC42 and the PAK family kinases, STE20 and PAK65 in yeast and mammals, respectively, that deliver signals to the nucleus (Figure 9). In addition, CDC42 must interact with effectors that directly impact the cytoskeleton. We have provided evidence that in neutrophils and lymphocytes this effector is WASP. In yeast and mammalian cells of nonhematopoietic origin the actin-regulating effectors for CDC42 are currently unknown. It is likely, however, that some of these effectors share properties with WASP.

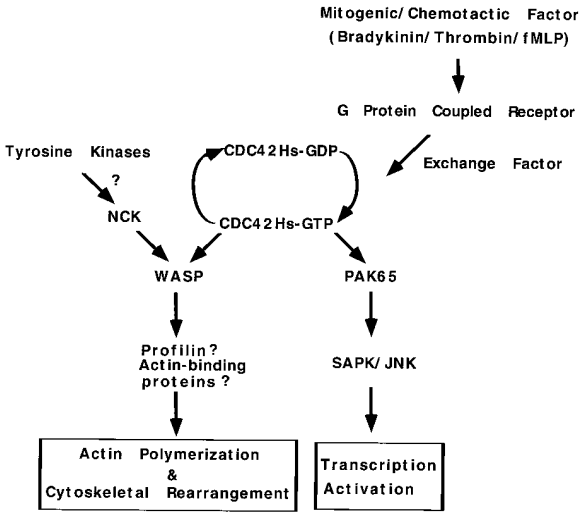

Figure 9. Role of WASP in Transmitting Signals from CDC42Hs to the Actin Cytoskeleton

In this model, CDC42Hs plays a pivotal role in delivering signals both to the nucleus via PAK65 and a MAP kinase cascade and to the cellular cytoskeleton via WASP. WASP may be targeted to particular cellular locations, possibly via its C-terminus, and then may recruit proteins such as profilin to nucleate new actin polymerization. There is also evidence that WASP may receive signals from tyrosine kinases via the adaptor protein NCK (Rivero-Lezcano et al., 1995). The upstream regulation of CDC42Hs may involve G protein–coupled receptors such as the bradykinin receptor (Kozma et al., 1995). In neutrophils, chemotactic receptors may feed into the CDC42Hs–WASP pathway, thereby producing the cytoarchitectural rearrangements necessary for cellular movement.

WASP contains polyproline regions that may interact with SH3-containing proteins as previously suggested (Derry et al., 1994, 1995b). Indeed, the SH3 domain of NCK was recently found to bind WASP (Rivero-Lezcano et al., 1995). NCK also interacts with another Rac/CDC42Hs effector protein, mouse PAK3 (Bagrodia et al., 1995), and with tyrosine kinase receptors (Li et al., 1992). This raises the possibility that WASP function may also be regulated by tyrosine kinase signaling pathways (Figure 9). In addition to the SH3-binding domain, WASP contains longer repeated proline-rich sequences, typically four to five consecutive residues with one stretch of 11 prolines. The role of repetitive proline sequences

is still uncertain. In several instances, they have been shown to be involved in association of multiple protein complexes (Williamson, 1994). Interestingly, we were able to identify in the database two additional sequences that contain a GBD consensus sequence and a proline-rich domain and are not related to PAK proteins. Both sequences encode long proline repeats, one of which reveals sequence homology to a vinculin-related protein from a lower organism (data not shown). It is interesting to note that the GBD has thus far only been found in PAK-related kinases and in proteins containing proline repeats, suggesting that these proline-rich proteins may represent novel GTPase targets that have similar functions.

We also identified two novel putative domains in WASP, WH1 and WH2, that are conserved among several proline-rich proteins. Some of these WH1/WH2-containing proteins were shown to be involved in the regulation of the actin cytoskeleton: Vasodilator-stimulated phosphoprotein (VASP) was shown to be implicated in actin polymerization (Haffner et al., 1995), and verprolin was shown to play a role in cytoskeletal organization and cellular growth in S. cerevisiae (Donnelly et al., 1993). In addition, ENA, the protein product of the Drosophila gene *enabled*, is involved in axonal architecture, consistent with a role in regulation of actin cytoskeleton (Gertler et al 1995). The fact that WH1 and WH2 are conserved among polyproline-rich proteins that are implicated in cytoskeletal organization suggests that they represent novel functional domains associated with the cytoskeleton. It is likely that the identified WH1/WH2-containing proteins may also be regulated by Rho-like GTPases via the interaction with a domain that is different from the putative GBD. It is tempting to speculate that WH1/WH2 domains may be involved in linking proline-rich proteins that are regulated by GTPases to structural components of the cytoskeleton.

Mutations in WASP that affect domains identified as having functional importance in this study have been found in WAS patients. The WH1 domain, encoded by exons 2 and 3, contains most of the missense mutations identified to date. Eleven consensus amino acids have been mutated in patients with either thrombocytopenia or classical WAS (Derry et al., 1994, 1995a; Kolluri et al., 1995; Zhu et al., 1995; Wengler et al., 1995; Kwan et al., 1995). The GBD, encoded by parts of exons 7 and 8, has been disrupted in a single patient with classical WAS (Derry et al., 1995a). In the C-terminal 59 amino acids, encoded by exons 10 to 12, missense mutations not involving the WH2 domain have been identified in patients with thrombocytopenia as well as with severe WAS, while mutations leading to premature termination of protein synthesis in this region are associated with severe disease (Derry et al., 1995a; Kolluri et al., 1995; Zhu et al., 1995; Wengler et al., 1995).

It is conceivable that WASP regulates the polymerization of actin in a similar mechanism as VASP. VASP was recently shown to interact with a monomeric actin-binding protein, profilin. VASP is implicated in the assembly of actin filaments, which are essential for the motility of Listeria monocytogenes in the cytoplasm of infected eukaryotic cells (Haffner et al., 1995; Reinhard et al., 1995; Theriot et al., 1994). The proline-rich motifs of VASP typically appear as $Gly(Pro)_5$, and three of the four copies are in a tandem repeat configuration. Moreover, a peptide corresponding to the VASP $Gly(Pro)_5$ tandem repeat competes with VASP in binding to profilin, supporting the notion that this region is important in VASP–profilin binding (Haffner et al., 1995). Interestingly, hWASP also contains a $Gly(Pro)_5$ sequence (Derry et al., 1994) while the mouse protein contains two such motifs (Derry et al., 1995b), suggesting that the effect of WASP on actin polymerization may be mediated through a direct interaction between WASP and profilin.

Three models have been proposed for the role of VASP/profilin in actin polymerization. In the first model, VASP-bound profilin may regulate actin polymerization by dissociating actin to increase the local concentration of free actin. In the second model, VASP and profilin interaction may lead to actin dissociation, followed by the formation of actin filaments. In the third model, VASP may be involved in stimulating the exchange of ATP on actin (Pollard, 1995; Theriot and Mitchison, 1993). In addition, VASP was detected at stress fibers, focal adhesions, and cell surface protrusions (Haffner et al., 1995), whereas the C-terminally truncated VASP failed to localize to these sites. In this study, we have provided evidence that the C-terminal 59 amino acids of WASP are involved in the formation of WASP clusters and actin polymerization. An alternative interpretation of our results is that the C-terminally truncated WASPΔC protein is not folded properly, thereby disrupting its normal function. However, if it is indeed the case that the C-terminal region of WASP is directly involved in actin polymerization, similar to the C-terminal region of VASP, these regions may contain domains necessary for localization of these proteins to distinct sites in the cell. The structural similarities between VASP and WASP suggest that they may play related roles in actin polymerization.

In summary, this study provides a link between the GTPase CDC42Hs, its effector WASP, and actin polymerization and demonstrates the pivotal role of CDC42 in the regulation of two pathways, a MAP kinase cascade leading to transcription activation and a second pathway linking to the cytoskeleton that in hematopoietic cells involves WASP (Figure 9). The elucidation of the biochemical mechanism by which WASP and CDC42Hs regulate actin polymerization will contribute to our understanding of the regulation of the actin cytoskeleton.

Experimental Procedures

Protein Purification and Peptide Sequencing

p62 was purified from human neutrophil cytosol by sequential chromatography steps, including Mono Q, phenyl–Superose, and Mono S columns, essentially as described previously for p65 (Martin et al., 1995). Partially purified p62 was electrophoresed on SDS–polyacrylamide gels, stained with Coomassie blue, and excised. The protein was digested with endoproteinase Ly-C, and peptides were separated by HPLC and were subjected to amino acid analysis as described previously (Martin et al., 1995).

Overlay Assay and Immunoblotting

The overlay assay used to detect CDC42Hs effectors is essentially as previously described (Martin et al., 1995). In brief, analyzed proteins were electrophoresed on an SDS–polyacrylamide gel followed by blotting to a PVDF membrane, washing, and blocking with BSA. Recombinant CDC42Hs (or other indicated GTPase) was preloaded

with [γ-^{32}P]GTP followed by 5–8 min incubation with the filter, 5 min wash, and 2 hr exposure to a film. The filter was then treated with 10% acetic acid, 50% methanol to dissociate the binding between CDC42Hs and the effector proteins and was reprobed sequentially with polyclonal anti-WASP antibody and with anti-hPAK65 antibody.

GST–WASP Expression Constructs and Polyclonal Antisera

A 825 bp MscI–XhoI fragment of the hWASP cDNA, corresponding to nucleotides 174–998, was cloned into the SmaI–XhoI site of pGEX-5X-3 (Pharmacia). This construct (pFusion3) was transformed into Escherichia coli, and a GST fusion protein corresponding to WASP amino acids 48–321 was purified using protocols supplied by the manufacturer. This protein was used to raise polyclonal antibodies in rabbits by standard procedures. The specificity of the antiserum was confirmed by Western blot analysis of protein extracts from WASP-expressing versus nonexpressing cells.

A deletion of amino acids 237–257 was introduced into the pFusion3 construct by PCR-based mutagenesis to generate GST–WASP without the GBD. The GST–GBD construct was made by PCR amplification of the region corresponding to amino acids 235–268 of the WASP cDNA with primers that contain the BamHI and EcoRI sites. The resulting fragment was ligated into the pGEX-5X-3 vector. Constructs were confirmed by DNA sequence analysis.

Epitope-Tagged Expression Constructs

pyDF30 (gift of D. Forentino, Howard Hughes Medical Institute, Stanford University) is a mammalian expression vector consisting of an SRα promoter (SV40 early promoter fused to RU5 sequences from HTLV-I LTR) upstream of a 16S splice region and polyadenylation signal between which lies a NotI cassette containing a FLAG epitope tag and restriction sites suitable for the insertion of cDNAs to produce N-terminal FLAG fusion proteins. pFLAG-N1 was constructed as follows: XbaI sites were introduced upstream of the WASP initiator methionine and in place of the WASP termination codon by using standard PCR mutagenesis. Thus, the entire hWASP cDNA could be cloned in-frame into the XbaI site of the vector pyDF30, downstream of the FLAG epitope tag. Sequencing of pFLAG-N1 indicated, however, that a single base deletion had occurred during construction that corresponds to a loss of a C-terminal nucleotide at position 1364 (Derry et al., 1994) and results in a frameshift and the generation of a stop codon at position 444. pFLAG-N1, therefore, produces a truncated protein lacking the last 59 amino acids of the mature protein (WASPΔC). The production of a truncated protein in transfected cells was confirmed by Western blotting with both an anti-FLAG M2 monoclonal antibody and the pFusion3 polyclonal antiserum. pFLAG-N2 was constructed by introducing NotI sites and a FLAG epitope tag by PCR mutagenesis, followed by replacement of the entire NotI cassette of pyDF30. pFLAG-N2 produces an N-terminal FLAG epitope tag fused to the entire 502 amino acid hWASP. This was confirmed by Western blotting. pEXV-Myc-Rac1V12 and pEXV-MycRac1N17 were previously described (Qiu et al., 1995a). pEXV-MycRhoAV14 and pEXV-MycRhoAN19 were described by Qiu et al. (1995b). pCMV-MycCdc42V12 and pCMV-MycCdc42N17 were gifts of M. Hart.

Transient Transfection and Immunofluorescence Photomicroscopy

Monkey COS7 and NRK epithelial cells (gift of L. Hein, Howard Hughes Medical Institute, Stanford University) were transfected with epitope-tagged WASP constructs via application of calcium phosphate–DNA coprecipitates to cells grown on coverslips. Jurkat T cells growing in suspension were transfected by electroporation and then cytospun onto microscope slides. After transfection, cells were cultured for 40–48 hr, fixed in 3% paraformaldehyde, permeabilized in 0.5% Triton X-100, and incubated with anti-FLAG mouse monoclonal antibody M2 (Eastman Kodak Company). Signal was detected with FITC-conjugated anti-mouse immunoglobulin antibody. Rhodamine-conjugated phalloidin (Sigma) was used to stain F-actin in dual-labeled experiments. Fluorescence photomicroscopy was carried out on a Zeiss Axiophot with appropriate filter sets for epifluorescence detection of FITC or rhodamine signals.

Microinjection and Immunofluorescence Procedures

PAE cells (gift of L. Claesson-Welsh, Ludwig Institute for Cancer Research) were grown in DMEM-F12 medium containing 10% fetal bovine serum. PAE cells were microinjected with plasmids in the nucleus, incubated for $\sim$5 hr, and fixed in 4% formaldehyde in Ca- and Mg-free PBS. Immunofluorescence procedures using primary antibodies against FLAG (Kodak) and Myc antibodies were carried out essentially as described by Stokoe et al. (1994). When indicated, cells were also stained with FITC–phalloidin (Sigma) at 0.5 μg/ml and by Texas red.

Acknowledgments

Correspondence should be addressed to A. A. We thank Maria Ruggieri, Gideon Bollag, and Heinz Furthmayr for critically reading the manuscript, Justin Hsuan and Nick Totty for excellent expertise in amino acid sequencing, Suzanne Pfeffer and Andy Wilde for advice on subcellular markers, Gaston Habets and Mike Freed for advice on BLAST searches, Matt Hart for the CDC42Hs-N17 plasmid, and Lena Claesson-Welsh for a gift of PAE cells. U. F. and J. M. J. D. were supported by the Howard Hughes Medical Institute, and V. L. by an N.I.H. Research Grant to U.F.

Received November 20, 1995; revised January 31, 1996.

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Note Added in Proof

While this paper was in the reviewing process, Aspenström et al. (1996), Curr. Biol. *6*, 70–75, reported that CDC42Hs binds to WASP.